

Research Note

Thermal Inactivation of H5N2 High-Pathogenicity Avian Influenza Virus in Dried Egg White with 7.5% Moisture

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ABSTRACT

High-pathogenicity avian influenza viruses (HPAIV) cause severe systemic disease with high mortality in chickens. Isolation of HPAIV from the internal contents of chicken eggs has been reported, and this is cause for concern because HPAIV can be spread by movement of poultry products during marketing and trade activity. This study presents thermal inactivation data for the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) in dried egg white with a moisture content (7.5%) similar to that found in commercially available spray-dried egg white products. The 95% upper confidence limits for *D*-values calculated from linear regression of the survival curves at 54.4, 60.0, 65.5, and 71.1°C were 475.4, 192.2, 141.0, and 50.1 min, respectively. The line equation $y = [0.05494 \times ^\circ\text{C}] + 5.5693$ (root mean square error = 0.0711) was obtained by linear regression of experimental *D*-values versus temperature. Conservative predictions based on the thermal inactivation data suggest that standard industry pasteurization protocols would be very effective for HPAIV inactivation in dried egg white. For example, these calculations predict that a 7-log reduction would take only 2.6 days at 54.4°C.

High-pathogenicity avian influenza virus (HPAIV) strains cause severe disease with high mortality in chickens and related gallinaceous poultry (14). In chickens, the initial replication site for HPAIV is the respiratory or intestinal tract, which is followed by systemic spread of the virus. During the 1983 to 1984 outbreak of HPAIV in the north-eastern United States, HPAIV was isolated from albumen, yolk, and the shell surface of eggs obtained from affected flocks in Pennsylvania (4). In one experimental study, HPAIV titers as high as 4.9 log mean embryo infective dose (EID₅₀) per ml of egg product were found in eggs laid by infected hens (13).

The presence of HPAIV in eggs is cause for concern because the virus can spread to susceptible poultry via the movement of infected poultry products. Due to the high economic cost of controlling HPAIV in poultry, the World Organization for Animal Health, the intergovernmental organization that establishes health standards for international trade of animals and animal products, recommends that poultry products from HPAIV-affected countries, zones, or compartments be treated to inactivate HPAIV prior to export (10). The demonstration of heat inactivation of avian influenza viruses in poultry products suggests that thermal processing could be an effective treatment for many products (8, 12, 13, 17, 18).

A previous study performed in our laboratory reported *D*-values for the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) in various egg products (13). Using this data, calculations were done to determine whether U.S. in-

dustry standards for egg product pasteurization, which were developed to inactivate contaminating *Salmonella* (6), are also sufficient for HPAIV inactivation. The calculations predicted that 15 days would be required to completely inactivate high titers of HPAIV in dried egg white at 54.4°C, rather than the 7 to 10 days specified by the industry standard. However, the moisture content of the freeze-dried egg white prepared for the previous study was not controlled, and was probably much lower than that in commercially available spray-dried egg white products. U.S. Department of Agriculture regulations state that the moisture content of spray-dried egg white must be greater than 6.0% for adequate destruction of *Salmonella*, and commercially available spray-dried egg white products typically have 6.5 to 8.0% moisture to provide adequate pathogen kill with an additional margin of safety (5).

For this study, HPAIV-contaminated freeze-dried egg white with an average moisture content of 7.5% was prepared and used for thermal inactivation experiments. A mathematical model for HPAIV inactivation in dried egg white was derived from survival curve data and used to predict the log reductions of HPAIV expected in dried egg white pasteurized according to standard industry protocols.

MATERIALS AND METHODS

Virus strain and preparation of virus-infected material.

Working stocks of the HPAIV strain A/chicken/PA/1370/83 (H5N2) (PA/83) were prepared by propagation in embryonating chicken eggs (15). All work with PA/83 HPAIV and with PA/83 HPAIV-infected materials was performed in U.S. Department of Agriculture-certified Biosafety Level 3-enhanced facilities.

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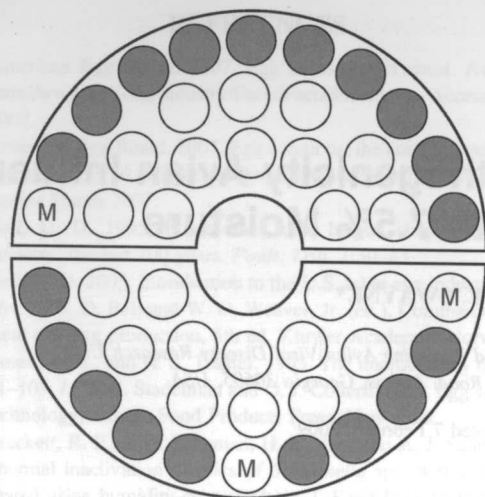


FIGURE 1. Serum vials containing liquid egg white with PA/83 HPAIV (gray circles) or no virus (white circles) placed in trays for freeze-drying. M, Samples for moisture analysis.

Preparation of samples for freeze-drying. Liquid egg white was prepared by mixing 0.2 g of spray-dried egg white (Michael Foods, Wakefield, NE) per milliliter of sterile deionized and distilled water. After mixing, approximately 200 ml of liquid egg white was filtered through sterile cheesecloth to remove any dried egg white that did not go into solution. For protocol development trials, no virus was added to the liquid egg white. For experiments with HPAIV, 1 ml of 8.1 log EID₅₀/ml of PA/83 HPAIV stock was added to 100 ml of liquid egg white, and no virus was added to the remainder. Sterile 5-ml serum vials (22 mm in diameter and 40 mm in height) were placed in drying trays, and 1.5 ml of liquid egg white was dispensed into each vial according to the pattern shown in Figure 1. Stoppers were placed in the vials in the "up" position used for freeze-drying. The vial trays were placed in sealed bags and frozen at -80°C for a maximum of 3 days before freeze-drying.

Freeze-drying liquid egg white containing HPAIV. Using samples with no virus added, the drying time and sample configuration required for reducing the moisture content of liquid egg white to 6.5 to 8.0% was determined empirically. This protocol was then adapted for drying liquid egg white containing HPAIV. Sample vials were freeze-dried in a MicroModulyo 1.5-liter freeze-dryer (Thermo Scientific, Waltham, MA). The drying chamber contained four vial-stoppering shelves stacked vertically. Two vial trays (Fig. 1) were placed on each of the two middle shelves. Chamber temperature was monitored by a thermometer placed on the top shelf. Vacuum was supplied by an E2M8 pump (Edwards, Wilmington, MA). After freeze-drying, the vials were sealed under vacuum and treated with 70% ethanol to remove any surface contamination. The vials from the inner and middle rows of each tray were discarded. The vials from the outer rows were capped with aluminum seals and then stored at -80°C . Four separate batches of dried egg white with HPAIV were prepared, one for each of the four temperatures used in this study. The batches used for 54.4, 60, 65.5, and 71.1 $^{\circ}\text{C}$ had HPAIV titers of 6.2, 6.2, 5.7, and 5.9 log EID₅₀/g, respectively.

Moisture analysis. For protocol development trials, samples for moisture analysis were prepared by combining the contents of two sample vials dried on the same shelf and in the same row. For freeze-drying runs with HPAIV, six negative-control vials were placed in the outer row. After freeze-drying, the weight of

the dry material in the negative-control vials was determined ($0.25 \text{ g} \pm 0.01 \text{ g}$, $n = 24$). Sample vials were paired based on freeze-dried sample weight (i.e., the two heaviest samples were combined) to obtain triplicate samples for moisture analysis. The flat end of a plunger taken from a syringe was used to grind the samples in the vials. Moisture determination was done by the AOAC International's vacuum-oven method for dried eggs (1), except that 0.5 g of sample was used for analysis. Each of the four batches of HPAIV-contaminated freeze-dried egg white that were prepared for thermal inactivation studies had a moisture content of 7.4 to 7.6% (maximum standard deviation of 0.5%), which is similar to that found in commercially available spray-dried egg white products.

Thermal inactivation procedure. Sample vials were completely submerged in a water bath (model 280 Precision Water Bath, Thermo Scientific) set to the target temperature. Water temperature and negative-control sample temperature were simultaneously monitored with a data logger thermometer (Omega, Stamford, CT). A needle was used to puncture a hole in the vial stopper and left in place so that a thermocouple wire could be threaded through, alongside the needle. Because the stoppers reseal after being punctured, the thermocouple wire was held securely in place when the needle was removed. To prevent or delay any leakage of water into the sample vial, silicone grease was applied around the insertion point. The thermocouple wire was positioned in the approximate geometric center of the sample. The $t = 0$ time point was set when the sample temperature was within 0.5°C of the water temperature (9). Instead of adjusting equations to account for heating lag time, the time required for samples to reach $t = 0$ at each temperature was determined before starting the thermal inactivation experiments. Because the heating lag time ranged from 5 to 10 min, regardless of temperature, $t = 0$ was set at 10 min for all temperatures. Therefore, $t = 0$ samples were removed from the water bath after a 10-min incubation, and timing for other samples started 10 min after the samples were placed in the water bath (e.g., samples at $t = 30$ min were placed in the water bath for a total of 40 min). Samples were immediately chilled on ice after removal from the water bath. Unless otherwise noted, triplicate samples were analyzed for each data point. The average HPAIV titers at $t = 0$ were 6.2, 6.2, 5.7, and 5.9 log EID₅₀/g for 54.4, 60, 65.5, and 71.1 $^{\circ}\text{C}$, respectively.

Virus isolation and titration. One and one-half milliliters of 4°C sterile phosphate-buffered saline was added to each sample vial. The samples were placed on ice for 30 to 60 min and allowed to dissolve, and then were gently mixed by swirling and pipetting. A 1:10 dilution series in brain heart infusion medium (Difco, Becton Dickinson, Sparks, MD) was prepared for each sample, and 1.5 ml of each dilution was inoculated into 9- to 11-day-old embryonating chicken eggs (15). Amnioallantoic fluid was harvested from eggs after a 2- to 3-day incubation and tested for hemagglutination activity (16). The 50% endpoints were calculated by the Reed-Muench method (19). The detection limit of the assay was 1.8 log EID₅₀/g.

Statistics and graphs. Statistical operations were performed with Sigma Stat, version 2.03 (1992 to 1997, SPSS, Inc., Chicago, IL). Graphs were prepared with Sigma Plot version 6.00 (2000, SPSS). Experimental D -values were calculated from linear regression of virus titer versus time at the given temperature ($D\text{-value} = -1/\text{slope}$). The upper limit of the 95% confidence interval for each experimental D -value was calculated from the following equation: $b_1 + t^* \times \text{SE}$, where b_1 is the slope coefficient, t^* is obtained from a Student's t test critical values table (two-tailed

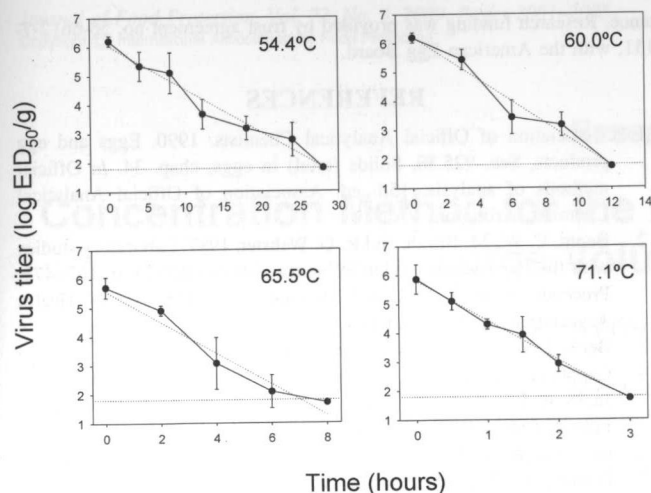


FIGURE 2. Survival curves for PA/83 HPAIV in dried egg white. Each data point represents the average titer of at least three samples, and the error bars indicate standard deviations. The detection limit of the assay is 1.8 log EID₅₀/g.

test, $\alpha = 0.05$), and SE is the standard error of the slope coefficient. The coefficients of determination (R^2) for the D -value graphs describe all of the data points rather than only averages. The z -value was calculated from linear regression of log D -values (expressed in minutes) versus the temperature (in degrees Celsius) (z -value = $-1/\text{slope}$). The upper limit of the 95% confidence interval for the z -value was calculated as described for the D -values, except that the following generic equation was used to calculate the upper limit of the 95% confidence interval for the slope coefficient: $b_1 + 2 \times \text{SE}$. For each D -value calculated from the z -value graph regression line equation, the upper limit of the 95% prediction interval for the D -value was calculated as follows: $y + [2 \times \text{RMSE}]$, where y is the predicted log D -value (in minutes), and RMSE is the root mean square error, or the standard error of the y estimate.

RESULTS

Survival curves and D -values for PA/83 HPAIV in dried egg white. Figure 2 shows survival curves for PA/83 HPAIV in dried egg white at 54.4, 60.0, 65.5, and 71.1°C. The R^2 and the D -values calculated from each survival curve are shown in Table 1. A linear model provided a fair-to-good fit for the survival curves, with R^2 values of 0.90 or higher for all temperatures except for 65.5°C. As shown in the 65.5°C graph (Fig. 2), the 4-h time point had an unusually large standard deviation. Similar results were obtained for a second set of triplicate samples. This variability probably accounts for the relatively low R^2 value for the 65.5°C curve. For each survival curve, the last data points include at least one sample in which PA/83 HPAIV was not detected: one of three samples for 54.4°C, two of three samples for 60°C, three of three samples for 65.5°C, and two of three samples for 71.1°C. Negative samples were graphed as 1.7 log EID₅₀/g, which is just below the detection limit of the assay (1.8 log EID₅₀/g). PA/83 HPAIV was not detected in additional samples incubated at 54.4°C for 2 days (three samples) or 3 days (nine samples).

TABLE 1. D -values for PA/83 HPAIV in dried egg white

Temp (°C)	D -value (min) ^a	R^2	95% PI upper limit for D -value (h) ^b
54.4	400.6	0.90	8.80
60.0	160.7	0.93	4.33
65.5	109.4	0.83	2.16
71.1	43.7	0.95	1.06

^a Experimental D -values calculated from the inactivation curves shown in Figure 2.

^b Upper limit of the 95% prediction interval (PI) for the D -value, calculated from the regression line equation + $[2 \times \text{RMSE}]$ (Fig. 3).

Regression line equation and the z -value. Figure 3 shows a linear regression plot of log D -value versus temperature for PA/83 HPAIV in dried egg white. The line equation and RMSE for the linear model are shown in the figure legend. A z -value of 18.2°C was calculated from the graph, with a 95% upper confidence limit of 23.0°C.

DISCUSSION

HPAIV has been isolated in eggs collected during natural outbreaks and experimental infection studies. During the 1983 to 1984 outbreak of H5N2 HPAI in the north-eastern United States, HPAIV was isolated from the internal contents of 22% of the chicken eggs sampled from flocks in Pennsylvania 1 to 18 days after clinical signs typical of HPAIV appeared, but the quantity of HPAIV in the samples was not determined (4). One report in the literature describes the isolation of 4.6 to 6.2 log EID₅₀/ml of H5N1 HPAIV from the internal contents of Japanese quail eggs collected during a 2003 to 2004 H5N1 HPAI outbreak in Thailand (11), demonstrating that HPAIV may be present in eggs from other susceptible avian species.

In studies with experimentally infected chickens, H5N2 HPAIV was not detected in eggs laid 1 to 2 days postinoculation, but was isolated from 88% of the eggs laid 3 to 4 days postinoculation (2). Some of these eggs had virus

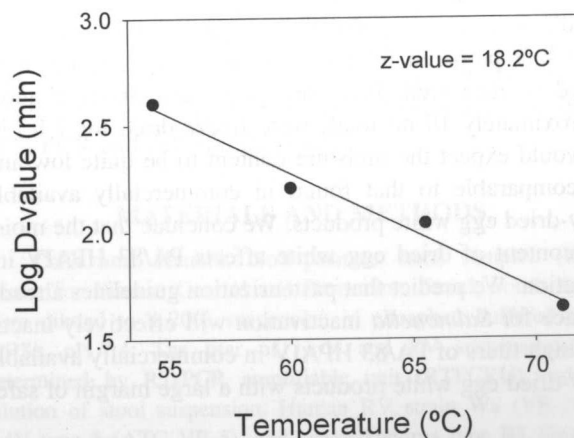


FIGURE 3. Linear regression plot of log D -value (in minutes) versus temperature (in degrees Celsius) for PA/83 HPAIV in dried egg white. Line equation: $y = [-0.05494 \times ^\circ\text{C}] + 5.5693$. RMSE = 0.0711, $R^2 = 0.98$.

titers greater than 4 log EID₅₀/ml (2), with a maximum titer of 4.9 log EID₅₀/ml (13). Liquid egg white is 87.81% water and the density of liquid egg white is 1.035 g/ml (6). Therefore, 1 ml of liquid egg white would be expected to yield about 0.136 g of dried product with 6.5 to 8.0% moisture. If no loss in virus titer occurred during the drying process, liquid egg white with 4.9 log EID₅₀/ml of HPAIV would yield dried egg white with 5.8 log EID₅₀/g of HPAIV. The dried egg white samples used in this study had 5.6 to 6.3 log EID₅₀/g of HPAIV and could therefore be considered "worst-case" samples. Based on the conservative *D*-value shown in Table 1, pasteurization at 54.4°C for 7 to 10 days would result in a 19.1- to 27.3-log reduction of HPAIV titer. A 7-log reduction, which would reduce an HPAIV titer of 6 log EID₅₀/g to 1 EID₅₀/0.1 g, would take only 2.6 days.

In contrast, a previous study done in our laboratory suggested that pasteurization at 54.4°C for 7 to 10 days would not be sufficient for inactivating high titers of HPAIV in dried egg white (13). The same PA/83 HPAIV strain was used for both studies, but the dried egg white used in the previous study had a titer of 7.3 log EID₅₀/g (3, 13). Our previous studies of HPAIV inactivation in chicken meat showed that HPAIV inactivation rates at 57°C were similar in meat samples, with HPAIV titers ranging from 3.7 to 8.0 log EID₅₀/g (17, 18). Therefore, differences in HPAIV titer are unlikely to account for the large differences in HPAIV inactivation rates measured during the dried egg white studies. Likewise, the results cannot be explained by differences in thermal inactivation methods. For the previous study, dried egg white samples were heated in thin-walled PCR tubes. The thin-walled tubes probably provided more efficient heat transfer to the sample, compared with the glass vials used in this study. If anything, this would result in faster, rather than slower, HPAIV inactivation rates. We conclude that the differences are most likely due to the moisture content of the dried egg white.

Moisture content is known to affect the thermal inactivation of pathogens in a variety of food products, including *Salmonella* in dried egg white (7). For this study, 1.5-ml aliquots of liquid egg white (138 ml total) were freeze-dried for 7 h and 45 min, resulting in a product with 6.5 to 8.0% moisture. The moisture content of the freeze-dried egg white prepared for the previous study was not controlled or measured. However, given that 80-μl aliquots (approximately 10 ml total) were freeze-dried for 7 h (3), we would expect the moisture content to be quite low and not comparable to that found in commercially available spray-dried egg white products. We conclude that the moisture content of dried egg white affects PA/83 HPAIV inactivation. We predict that pasteurization guidelines already in place for *Salmonella* inactivation will effectively inactivate high titers of PA/83 HPAIV in commercially available spray-dried egg white products with a large margin of safety.

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